

BURGETT, KYLE D., M.S. Studies of Alpha Kinase 1 and Its Potential to Influence Cytoskeletal Reorganization in *Dictyostelium discoideum*. (2016)  
Directed by Dr. Paul Steimle. 55 pp

Maintaining and remodeling the actin cytoskeleton is a critical part of normal cellular functioning for all eukaryotes, as it is crucial for cell movement, endocytosis, exocytosis, and maintaining cellular morphology. Within the social amoeba *Dictyostelium discoideum*, myosin II associates with the actin cytoskeleton and is directly responsible for the contraction of opposing actin filaments. Cytoskeletal remodeling is achieved by cycling myosin II between a bipolar filamentous form and a monomeric form. In *Dictyostelium*, the family of alpha kinases known as myosin heavy chain kinases (MHCKs) has been shown to be the primary regulators of myosin II bipolar filament turnover. By phosphorylating specific threonine residues on the tail region of the myosin II protein, MHCKs control the assembly/disassembly of myosin II filaments. Alpha Kinase 1 (AK1) represents a previously uncharacterized member of the MHCK family in *Dictyostelium*. The data presented in this thesis provides evidence that AK1 can influence myosin II bipolar filament turnover and indeed act as a myosin II heavy chain kinase. Overexpression of AK1 yields a myosin II null phenotype, characterized by cytoskeletal defects arising from elevated levels of phosphorylated monomeric myosin II, as evidenced by 1) an inability to complete cytokinesis in suspension culture, and 2) delayed multicellular development. Localization studies performed with GFP-tagged AK1 reveal a localization pattern that is consistent with a role for AK1 in vesicle trafficking, unique amongst the MHCKs. Taken together, these data support the hypothesis that AK1 can function as a MHCK in *Dictyostelium* and suggest that more detailed biochemical and

cellular studies of AK1 are warranted to fully characterize this unique protein and its potential role in regulating myosin II activity.

STUDIES OF ALPHA KINASE 1 AND ITS POTENTIAL TO  
INFLUENCE CYTOSKELETAL REORGANIZATION IN  
*DICTYOSTELIUM DISCOIDEUM*

by

Kyle D. Burgett

A Thesis Submitted to  
the Faculty of The Graduate School at  
The University of North Carolina at Greensboro  
in Partial Fulfillment  
of the Requirements for the Degree  
Master of Science

Greensboro  
2016

Approved by

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Committee Chair

## APPROVAL PAGE

This thesis written by Kyle D. Burgett has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair \_\_\_\_\_

Committee Members \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_  
Date of Acceptance by Committee

\_\_\_\_\_  
Date of Final Oral Examination

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## CHAPTER I

### INTRODUCTION

#### **An Introduction to *Dictyostelium discoideum***

*Dictyostelium* is an amoeboid unicellular eukaryote existing naturally in moist soil and leaf litter where it acts as a phagotropic bacterivore. Under stressful conditions, such as low food availability, *Dictyostelium* enters into a complex developmental cycle containing three distinct stages: aggregation, migration, and culmination. During development, *Dictyostelium* transitions from a free-living, single-celled organism into a multicellular migratory slug and ultimately into a fruiting body containing spores for the next generation (Figure 1). These transitions are directly correlated with food availability. Thus, when food is scarce, *Dictyostelium* will aggregate and develop into an intermediate migratory slug stage which can seek out new, food-rich areas through chemotaxis, thermotaxis, or phototaxis (Kessin, 2001).

The ability to undergo multicellular development is one of several important features that makes *Dictyostelium* an attractive model organism to study directed cell migration and chemotaxis. During the early stages of development, *Dictyostelium* cells aggregate and form a mound structure. Aggregation is driven primarily by cyclic adenosine monophosphate (cAMP) signaling, in which a founder cell releases pulses of cAMP, which bind to cAMP receptors on the surfaces of surrounding cells. Receptor binding by cAMP leads to the activation of G-protein coupled pathways and ultimately



results in cells taking on an extended and polarized shape that facilitates migration toward the source of cAMP. Stimulated cells are then able to secrete cAMP as well, thus propagating the cAMP wave initiated by the founder cell and establishing a cAMP concentration gradient. Cells will move along this gradient towards the founder cell for up to 60 seconds and then stop until the next secretion (Figure 2). This allows for directed cellular migration, affecting many cells at once in a simple yet tightly controlled process (Kessin, 2001).

Many of the cellular processes that occur in *Dictyostelium* are analogous to those found in higher eukaryotes. *Dictyostelium* also exists as a haploid organism that is capable of expressing eukaryotic plasmids. This makes genetic manipulation of the organism relatively simple and readily accessible (Kessin, 2001). The relative ease with which *Dictyostelium* can be grown and maintained in a laboratory setting, combined with its unique developmental cycle, are what ultimately make *Dictyostelium* a valuable model organism for chemotactic research.

## **Myosin II and Cytoskeletal Remodeling**

The *Dictyostelium* genome contains 34 Mbp spread across six separate chromosomes. Of the approximately 10,000 genes in the genome, there are twelve genes that encode different myosin isoforms. Seven of these encode myosin I proteins, while only one gene encodes for myosin II (the myosin II heavy chain). Myosin is an ATP-dependent motor protein found in eukaryotes and is responsible for actin-based motility. Myosin II is hexameric protein comprised of two 16-kDa essential light chains, two 18-

kDa regulatory light chains, and two 243-kDa heavy chains (Figure 3). In *Dictyostelium*, myosin II associates with the actin cytoskeleton and is directly responsible for the contraction of opposing actin filaments. If the actin filaments undergoing contraction are tethered to the plasma membrane then contraction of the cell will occur. The globular head region of the myosin II protein uses the energy derived from ATP hydrolysis to govern the contraction of these actin filaments (De la Roche & Cote, 2001). This is activated via phosphorylation of the regulatory light chain subunits of the myosin II molecule. In addition, myosin II-mediated contraction of actin filaments requires that myosin II be assembled into bipolar filaments. Bipolar filament assembly is inhibited by phosphorylation of three specific threonine residues in the myosin II heavy chain. (Yumura et al., 2005).

Cytoskeleton remodeling is an important mechanism required for normal eukaryotic cell functioning. The actin cytoskeleton provides a rigid, but dynamic, framework for the cell, and remodeling of the cytoskeleton is important for cellular movement, protein secretion, and maintenance of cellular morphology. Cellular migration is essential to proper functioning of the immune system of almost all multicellular organisms. Macrophages, a key component of our own immune systems, utilize chemotaxis to track down and subsequently phagocytose invading pathogens. Patients suffering from certain syndromes, such as AIDS or other genetic predispositions, display markedly decreased and inefficient levels of macrophage migration (Smith et. al., 1984).

Cytoskeletal remodeling is also of particular importance in cells undergoing epithelial -mesenchymal transition (EMT). EMT is required for the proper functioning of

multicellular organisms whose cells are undergoing complex processes such as wound-healing (Kalluri & Weinberg, 2009), embryonic development (Hay, 2005), and the female menstrual cycle (Ahmed et. al., 2006). It is also important to the initiation of metastasis and the subsequent development of the cancer phenotype (Kalluri & Weinberg, 2009). A better understanding of the signaling pathways and regulatory components associated with cytoskeletal remodeling, as well as the factors which drive these processes, can lead to a better understanding of how to treat defects within these pathways should they arise.

### **Myosin Heavy Chain Kinases in *Dictyostelium***

In order for myosin II to contract actin filaments, and therefore affect cell shape, it must first assemble into bipolar filaments. In *Dictyostelium*, the family of alpha kinases known as myosin heavy chain kinases, or MHCKs, has been shown to be the primary regulators of myosin II bipolar filament turnover (Figure 4). Most of the MHCKs within this family of alpha kinases share a similar structure to one another. All of them contain an alpha kinase catalytic domain as well as a C-terminal WD repeat domain. MHCK A and D both share a coiled-coil region in addition to these other two domains (Figure 5). Each of these kinases has been demonstrated to localize to specific parts of the cell during chemotaxis (Steimle et al., 2001). MHCK A localizes to the leading edge of the cell during chemotaxis (Kolman et al., 1996), whereas MHCK C localizes to the rear of a migrating cell (Nagasaki et al., 2002) and to the cleavage furrow in the latter stages of cell division, suggesting a role in the removal of Myosin II as the furrow progresses

(Liang, Wenchuan, et al, 2002). Disruptions of either of these MHCKs result in chemotactic defects. MHCK B displays a more diffuse localization pattern and overexpression leads primarily to defects in cytokinesis (Rico and Egelhoff, 2003).

Alpha Kinases themselves are a unique family of proteins in that they contain little sequence similarity to that of known kinases and are able to phosphorylate both threonine and serine residues in the context of alpha helices. This is in comparison to conventional protein kinases, or CPKs, which are able to phosphorylate amino acid residues in the context of irregular structures or loops (De la Roche & Cote, 2001). Even though recent studies have demonstrated the ability of certain alpha kinases to phosphorylate amino acids outside of the context of an alpha helix, the name has remained. Many alpha kinases also come paired with other types of protein domains such as WD-repeat domains, Willebrand factor-like motifs (vWKa) and cation channels such as TRPM6 or TRPM7. Both conventional protein kinases (CPKs) and atypical protein kinases (APKs) have been shown to be widely distributed throughout prokaryotes and eukaryotes. Alpha kinases, however, have only ever been found in eukaryotes, suggesting that they are a relatively recent evolutionary adaptation (Middlebeek et al., 2010). It has been hypothesized that alpha kinases evolved within eukaryotes to facilitate a more complex means of signal transduction within higher-functioning multicellular organisms (Scheef & Bourne, 2005). As such, it follows that alpha kinases would play an important part in human signal transduction, and to date 6 unique alpha kinases have been discovered in humans, including kinases such as kidney alpha-kinase, lymphocyte alpha-kinase, muscle alpha-kinase, and heart alpha-kinase. An alpha kinase which directly

phosphorylates myosin II within human cells has yet to be discovered, and has been shown to be absent in most higher-level eukaryotes such as fungi, yeast and certain yeast and plants (Drennan & Ryazanov, 2004).

### **Alpha Kinase 1 – A Novel Protein**

Among the alpha kinases in *Dictyostelium* only the protein Alpha Kinase 1 (AK1) has yet to be characterized in any way. Alpha Kinase 1 (Figure 6) is an atypical protein kinase containing 1,352 amino acids and weighing 154 kDa, and is encoded by the DDB\_G0292150 gene in *Dictyostelium*. AK1 contains an unusually high number of polyQ repeats which comprise 22.8% of the entire protein structure (Figure 6). Although polyQ regions can be found among other members of the MHCK family, the level at which they are present within the Alpha Kinase 1 protein is unique and could speak to a deeper function. Alpha Kinase 1 contains only two known domains, both of which have been inferred by sequence homology: a C-terminal Alpha kinase domain and an N-terminal ARF-GTPase (ARF-GAP) domain (Yumura et al., 2005). The kinase catalytic domain shares homology with the catalytic domains found in the other MHCKs, suggesting a possible role in regulating myosin II filament assembly/disassembly. The ARF-GAP region could induce hydrolysis of ARF-bound GTP, thereby affecting membrane trafficking and cellular remodeling, as ARFs have been shown to be associated with actin (Randazzo and Hirsch, 2004). As there is only one known ARF protein in *Dictyostelium*, this could also suggest involvement in the regulation of cytoskeleton structure.

Knowing this information, I would like to examine the possible means by which Alpha Kinase 1 could influence cytoskeletal reorganization in *Dictyostelium discoideum*.

## CHAPTER II

### HYPOTHESIS AND SPECIFIC AIMS

I hypothesize that the novel protein Alpha Kinase 1 can function as a myosin II heavy chain kinase and regulate myosin II filament turnover in the model organism *Dictyostelium discoideum*. To this end, I focused on characterizing the *in vivo* effects of over-expressing AK1 protein on myosin II-dependent process such as cytokinesis and multicellular development. To gain further insight into AK1 function, I also generated a GFP-tagged AK1 fusion protein to ascertain cellular localization *in vivo*.

1. **Cellular characterization of the AK1 protein.** AK1 function was explored *in vivo* by performing experiments examining the effects of elevated AK1 expression on *Dictyostelium* cytokinesis and multicellular development. Overexpression of AK1 is expected to mimic a constitutively activated state and any resulting changes in cellular phenotype should reveal which processes are influenced by the kinase. For example, previous studies have shown that overexpression of any of the of the *Dictyostelium* MHCKs leads to a myosin II null phenotype in which cells exhibit severe defects in cytokinesis and multicellular development. This phenotype is consistent with the role of the MHCK in phosphorylating the MHC and driving bipolar filament disassembly. In cells overexpressing an MHCK, there will be elevated levels of phosphorylated myosin II that are unable to form contraction-competent myosin II filaments.

My studies of AK1 overexpression involved selecting *Dictyostelium* cells with high copy numbers of the plasmid for expression of FLAG-tagged AK1. This was achieved by selecting cells at higher and higher concentrations of G418, so that only cells containing greater numbers of the plasmid, which contains a gene for resistance to G418, were able to grow. In addition to having elevated levels of the G418 resistance gene product, these cells were also expressing elevated levels of plasmid-encoded AK1 protein. The level of AK1 overexpression was examined via immunoblot analysis of lysates (using anti-FLAG antibody) of *Dictyostelium* cells selected at 10 µg/ml G418 and at 50 mg/ml G418. Cell lines with confirmed over expression of AK1 were then examined for defects in cytokinesis by examining cell growth rates and morphology when cultured in suspension. Defects in cytokinesis, especially those resulting from alterations in myosin II activity, are often manifested as a decrease in growth rate associated with an increase in cells size and multinuclearity. These cells were also examined for defects in multicellular development. These may be manifested in a number of ways, including termination at the mound stage (associated with myosin II defects) or small fruiting body formation.

- 2. Localization studies of the AK1 protein.** Studies of the other Alpha Kinase family members in *Dictyostelium* (MHCKs A, B, C, and D) have revealed distinct localization patterns of the various MHCKs within resting and dividing cells as well as cells undergoing chemotaxis (Steimle et al., 2001). For example, green



fluorescent protein (GFP) tagged MHCK A and MHCK C localize to the front and rear of migrating cells, respectively. Recent studies carried out in our lab (Steimle, unpublished) have revealed that MHCK D exhibits similar localization properties as MHCK A, with a strong enrichment at that front end of migrating cells. For the second aim of my thesis project, I examined the localization of AK1 by expressing the protein with an amino terminal GFP tag in *Dictyostelium* cells. Expression of the fusion protein was confirmed visually via fluorescence microscopy and immunoblot analysis. The primary goal of these localization studies is to gain insight into the potential function of AK1 during the highly dynamic processes of cell migration and cytokinesis.

## CHAPTER III

### MATERIALS AND METHODS

#### **Gene Synthesis and Cloning into *Dictyostelium***

Gene synthesis was carried out by Genscript. The full length AK1 gene was inserted into the *Dictyostelium* expression vector pTX-FLAG for the expression of AK1 as with an amino-terminal FLAG-tagged in *Dictyostelium* (Figure 7A).

For protein purification and overexpression studies, *Dictyostelium* clones containing recombinant plasmid with the AK1 gene were selected. High-level and constitutive expression of Flag-tagged AK1 is driven by the actin-15 promoter that sits upstream of the *ak1* gene (Levi et al., 2000). Wildtype *Dictyostelium* Ax2 cells were transformed with the recombinant pTX-FLAG vector (with *ak1* gene) via electroporation using the following protocol. All cell lines were created using the following methods: *Dictyostelium* cells were allowed to grow in HL5 medium (Dextrose 14g/L, Oxoid yeast extract 7g/L, Oxoid proteose peptone 14g/L, Na<sub>2</sub>HPO<sub>4</sub>•7H<sub>2</sub>O 0.5g/L, KH<sub>2</sub>PO<sub>4</sub> 0.5g/L, H<sub>2</sub>O 1000mL) until confluent. Cells were then collected, counted using a hemocytometer, and brought to a final concentration of 1 x 10<sup>7</sup>/mL. They were then transferred to a Falcon tube and centrifuged at 2500 rpm for 5 minutes. The pellet was then washed with 1mL H50 buffer (20mM HEPES, 50 mM KCl, 10mM NaCl, 1mM MgSO<sub>4</sub>, 5mM NaHCO<sub>3</sub>, and 1mM NaH<sub>2</sub>PO<sub>4</sub>) and centrifuged again at 2500 rpm for 5 minutes. This step was repeated once more, after which the pellet was resuspended in 300μL of H5

buffer. This volume was then distributed amongst 0.1 cm Bio-Rad Gene Pulser cuvettes by adding 100 $\mu$ L to each cuvette. 1 $\mu$ L of plasmid was added to two of the cuvettes, keeping one of them as a negative control. The cuvettes were then pulsed twice at 0.85 kV/25mF and allowed to recover on ice in HL5 medium. After 5 minutes, the cells were then transferred to 10cm Petri dishes containing 10mL of HL5 medium. The following day, cells were selected for using G418 expression, as the pTX vector contains a G418 resistance coding region. Because resistance to G418 will be increased depending on the number of copies of the pTX plasmid that the cell contains, selection for colonies expressing higher levels of protein was performed using increasingly higher concentrations of G418 (up to 50 $\mu$ g/mL max).

### **Preparation of Cell Lysates**

To examine recombinant AK1 protein expression, cell lysates were prepared as follows: Cells expressing resistance to G418 were collected at a concentration of  $5 \times 10^7$  cells and transferred to a 15mL Falcon tube wherein they were pelleted at 2500 rpm for 5 minutes. The pellet was then washed in 1mL of 10mM Tris pH 7.5 and centrifuged again at 2500rpm for 5 minutes. This wash step was repeated two more times. Afterwards, the pellet was placed on ice. The pellet was then resuspended in 1mL ice cold H50 buffer (20mM Hepes, 50mM KCl, 10mM HCl, 1mM Mg<sub>2</sub>SO<sub>4</sub>, 5mM NaHCO<sub>3</sub>, 1mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0) and pelleted again at 2500 rpm for 5 minutes. This wash step was repeated twice and on the last time the supernatant is discarded and the pellet was placed on ice. The pellet was then resuspended in 100 $\mu$ L lysis buffer (1.5mL 1M Tris pH 8.0, 0.27g NaPPi,

0.3mL 0.5M EDTA, 57mg EGTA, 3mL 20% Triton X-100,) supplemented with protease inhibitors (2mg/mL antipain, 10 KIU/mL aprotinin, 10mg/mL benzamidine, 1mg/mL leupeptin, 5mg/mL Pefabloc, 10mg/mL L-1-chloro-3[4-tosylamido]-7-amino-2-heptanone-HCl N- $\alpha$ -tosyl-L-lysine chloromethyl ketone [TLCK] in ddH<sub>2</sub>O) followed immediately by the addition of 100 $\mu$ L 5x SDS sample buffer (50mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% mercaptoethanol, 12.5mM EDTA, 0.02% bromophenol blue), preheated to 95°C. The sample was briefly vortexed and then heated at 95°C in a heating block for 4 minutes. Samples were allowed to cool before loading on SDS-PAGE gel. Remaining samples were stored at -20°C.

### **SDS-PAGE Gels and Western Blot Analysis**

All samples were run on 6% SDS-PAGE (4.2mL ddH<sub>2</sub>O, 1.6mL 30% acrylamide, 2mL 1.5M Tris pH 8.8, 80 $\mu$ L 10% SDS, 80 $\mu$ L 10% APS, 8 $\mu$ L TEMED) with 6% stacking gel (2.6mL ddH<sub>2</sub>O, 1mL 30% acrylamide, 1.25mL 0.5M Tris pH 6.8, 50 $\mu$ L 10% SDS, 50 $\mu$ L 10% APS, 5 $\mu$ L TEMED).

AK1 protein expression was determined via Western Blotting. Samples were loaded onto a 6% SDS-PAGE gel and placed in a chamber filled with 1x running buffer (25mM Tris-Base pH 8.8, 90mM Glycine, 0.1% SDS) set to run at 70mA for approximately 45 minutes or until the dye front reached the bottom of the gel. The gel was then placed inside a transfer cassette with Immobilon transfer membrane and set in a transfer chamber filled with transfer buffer (2.9g/L glycine, 5.8g/L Tris base, 0.37g/L SDS, pH 8.3), a stir bar, and an ice pack and then run at 300mA for 1 hour. Afterwards,

the transfer membrane was removed and allowed to incubate in blocking solution (5mL TBST, 2.5% BSA) overnight with rotation at 4°C. The following day, the blot was probed with primary antibody (2.5µL of rabbit anti-FLAG in 5mL of TBST) for 1 hour with rotation at room temperature. The blot was then washed three times with fresh TBST for five minutes each with agitation. The blot was then probed with secondary antibody (2µL of goat anti-rabbit in 5mL of TBST) for 1 hour at 4°C. Afterwards, the blot was again washed three times with TBST for five minutes each with agitation. Following the washes, 500µL of Advanta WesternBright ECL was added to the membrane and allowed to incubate at room temperature for 2 minutes at which time excess solution was drawn off. The blot was then imaged using a Bio-Rad ChemDoc system.

### **Protein Purification from *Dictyostelium***

Clones of *Dictyostelium* cells that were selected at 50 µg/ml G418 for high plasmid copy number (and resulting over-expression of AK1 fusion protein) were used for protein purification. Cells were collected from four plates and combined in a 50mL Falcon tube. They were pelleted at 2500 rpm for 10 minutes and the supernatant discarded. The pellet was washed twice with 10 mL wash buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1x protease inhibitors) and finally placed on ice while 250 µL of magnetic anti-FLAG beads were washed. The bead slurry was placed in a 1.5mL tube and 1mL wash buffer was added. It was then placed in a magnetic tube holder where the resulting supernatant was drawn off and replaced by fresh wash buffer. This

process was repeated 10 times using 1mL of wash buffer each time. After 1 hour, the tube was retrieved and the supernatant drawn off. The beads were then incubated with 250µL FLAG elution buffer (1mg/mL FLAG peptide, 24 µM PMSF, 1.0 mM EDTA, 2 mM DTT) supplemented with 1x protease inhibitors and allowed to incubate for 30 minutes at 4°C with rotation. The tube was retrieved, placed back into the magnetic column, and the eluate (containing the purified fusion protein) was collected. All samples were analyzed via 6% SDS-PAGE as previously described.

### **Analysis of AK1 Phosphorylation State**

To examine the phosphorylation state of purified AK1, the protein was phosphatase treated (Antarctic phosphatase, NEB) and then examined for any resulting changes in electrophoretic mobility of the purified protein. A 20 µL reaction mix was prepared containing 2 µL of 10x Antarctic Phosphatase Reaction Buffer (50 mM Bis-Tris-Propane-HCl, 1 mM MgCl<sub>2</sub>, 0.1 mM ZnCl<sub>2</sub>, pH 6.0), 1 µL of Antarctic Phosphatase, 5 µL of purified protein, and 12 µL of dH<sub>2</sub>O. The reaction mix was incubated at 37°C for 30 minutes at which point the reaction was stopped through heat-inactivation at 80°C for 2 minutes. Samples were then analyzed via 6% SDS-PAGE as previously described.

### **Growth Assays in Shaking Culture**

To assay for the effects of AK1 overexpression on the ability of *Dictyostelium* cells to divide in suspension culture, cells overexpressing the AK1 protein were collected from confluent plates and counted via flow cytometry. Each cell line was then used to

inoculate 25 cm<sup>2</sup> Corning flasks (5 x 10<sup>4</sup> cells/ml) containing 30 mL HL5 supplemented with appropriate concentrations of G418 (10, 35, or 50 µg/ml). Cultures were then left to incubate at room temperature with shaking at 200 rpm for seven days and counted every day (except within the first 48 hours). Cell counts were analyzed and graphed in Microsoft Excel. Ax2 wild type cells as well as cells expressing only a FLAG-tagged plasmid grown in G50 were used as controls. The experimental cell lines were colonies grown in G10, G35, and G50.

### **Detection of Multi-Nucleation in Cell Lines Overexpressing AK1**

Cell lines with overexpression of the AK1 protein were collected from confluent plates and counted via flow cytometry. Each cell line was then used to inoculate 25cm<sup>2</sup> Corning flasks containing 30mL HL5 supplemented with appropriate concentrations of G418 at a concentration of 5x10<sup>4</sup> cells/mL. The cells were collected after 72 hours in suspension culture and seeded on glass slides at a concentration of 1x10<sup>4</sup> cells/mL. The growth medium was removed, allowing the cells to settle to the bottom of the slide where they were then fixed using ice cold methanol. The cells were allowed to incubate for 5 minutes and then washed using 1x Tris Buffered Saline (1x TBS: 10ml 1M Tris-HCl, 8g NaCl, 0.2g KCl, & dH<sub>2</sub>O). Nuclei were stained using 300nM 4,6-diamidino-2-phenylindole (DAPI) for 5 minutes and washed afterwards with starvation buffer. The number of nuclei per cell were then counted using microscopy. All data was then analyzed via Microsoft Excel. Ax2 wild type cells as well as cells expressing only a

FLAG-tagged plasmid grown in G50 were used as controls. The experimental cell lines were colonies grown in G10, G35, and G50.

### **Analysis of Development and Stalk Length**

Cells were collected from confluent plates and counted via flow cytometry. They were then plated onto starvation plates (starvation buffer supplemented with 15g/L agar) at a concentration of  $1 \times 10^7$  cells/mL and grown at 22°C for 48 hours. Images were taken every 4 hours. Ax2 wild type cells as well as cells expressing only a FLAG-tagged plasmid grown in G50 were used as controls. The experimental cell lines were colonies grown in G10, G35, and G50.

In order to analyze average stalk lengths for each cell line, after 48 hours the agar from each plate was removed and cut into 2-3mm segments. These segments were laid sideways onto glass slides and then imaged, taking care to not adjust the focal plane of the microscope. These images were then analyzed using the ImageJ software developed by the NIH. In order to control for the varying depth that each stalk could be at on each agar segment, only stalks that were in the same focal plane were measured. These data were then analyzed via Microsoft Excel.

### **AK1 Localization Studies**

Gene synthesis was again carried out by Genscript. The full length AK1 protein was synthesized into a pTX-GFP plasmid for expression as a GFP-tagged fusion protein within *Dictyostelium* (Figure 7B). Plasmid was cloned into *Dictyostelium* utilizing the



same protocol as outlined above (see **Gene Synthesis and Cloning into *Dictyostelium***). Cells expressing the AK1 pTx-GFP plasmid were selected for using G418. These cells were collected and resuspended in starvation buffer (0.5M MES pH 6.8, 1.0M CaCl<sub>2</sub>, 1.0M MgSO<sub>4</sub>, dH<sub>2</sub>O) and seeded onto a glass slide at a concentration of 5x10<sup>4</sup> cells/mL for fluorescence microscopy. Excess HL5 was removed by washing with starvation buffer. Localization of the AK1-GFP protein was visualized in vegetative, dividing, and chemotaxing cells using an Olympus IX70 microscope system and images were processed using Image Pro-Plus software. Chemotaxis was induced by incubating cells in starvation buffer for 2-8 hours before visualization and then adding 10mM cAMP to the plate while images were being taken. Images were taken every 10 seconds unless otherwise noted. Latrunculin A was administered at a concentration of 100ng/mL and cells were allowed to incubate for 30 minutes prior to imaging. Jasplakinolide was added at a concentration of 10μM and allowed to incubate for 5 minutes prior to imaging. DMSO was used as a control in both assays.

## CHAPTER IV

### RESULTS

#### Western Blot Analysis of Alpha Kinase 1

Alpha Kinase 1 was expressed in *Dictyostelium* as a FLAG-tagged fusion protein through transformation with pTx-FLAG plasmid containing an AK1 insert. In order to confirm proper protein expression, a Western Blot was run using lysates prepared from the cell lines transformed with this pTx-AK1-FLAG vector. AX2 wildtype cells were used as controls. The experimental cell lines used were G10, G35, and G50 cells lines. These names correspond to the levels of G418 that they are able to grow in (10 $\mu$ g/mL, 35 $\mu$ g/mL, and 50 $\mu$ g/mL) and directly correlate with protein expression. By selecting for higher levels of antibiotic resistance, I was able to isolate clones containing higher plasmid copy numbers and therefore elevated levels of the AK1 protein. Probing with an anti-FLAG antibody reveals expression of a FLAG-tagged protein with a molecular weight of approximately 215 kDa with very little breakdown product (Figure 8A).

The same analysis was carried out after affinity purification of the protein was performed and a strong band at approximately 215 kDa can again be seen with very little breakdown product (Figure 8B). The expected size of the AK1 protein according to the Dictybase Stock Center is 154 kDa. This observed size is significantly higher than expected but may be due to a variety of post-translational modifications that can affect the mass-to-charge ratio of the protein. Phosphorylation, one such modification, is a

prime suspect considering the similarity of Alpha Kinase 1 to other MHCKs that are known to auto-phosphorylate.

### **Assay for Phosphorylation of Alpha Kinase 1**

In order to investigate if the aberrant migration of Alpha Kinase 1 was due to phosphorylation, samples were incubated with Antarctic Phosphatase and then analyzed via Western Blot (Figure 8C). The analysis revealed that incubation with Antarctic Phosphatase did not significantly alter the migration position of Alpha Kinase 1 as compared with pre-incubation. This indicates that phosphorylation is not a post-translational modification of AK1. However, this does not rule out the ability of AK1 to autophosphorylate, nor does it rule out other post-translational modifications that may be attributing to the aberrant running size.

### **Analysis of Cell Lines for Myosin II Null Phenotype**

Cells that are overexpressing an MHCK grow poorly when grown in suspension culture and produce a multinucleated phenotype, as cytokinesis is disrupted. I expected that overexpression of AK1 might produce a similar phenotype if it is acting as an MHCK. To this end, a shaking growth culture assay was performed using three different cell lines: G10, G35, and G50. AX2 wildtype cells and cells containing an empty pTX-FLAG vector were used as controls. Analysis of the data gathered from suspension culture assays reveals a negative correlation between average growth rates and increasing expression of the AK1 protein, as would be expected if the cells were displaying a

Myosin II null phenotype (Figure 9A). The FLAG control did grow slower than the AX2 control, but they did eventually attain a level of growth equal to 70% that of the AX2 (Day 8). By comparison, the G50 cell lines were only able to reach 0.34% of the growth of the AX2 (Day 2). There is also a clear negative correlation between AK1 expression levels and viability of these cell lines (Figure 9B). As expected, staining with DAPI also reveals significant amounts of multi-nucleation within cell lines overexpressing AK1 (Figures 10A and 10B). The average nuclei per cell shows a nearly 60% increase in the G50 cell lines over the AX2 control.

Images obtained from developing cell lines overexpressing AK1 show significant differences from the AX2 wild type control as well as the FLAG vehicle control (Figure 11A). Cell lines expressing AK1 protein in large quantities display markedly delayed aggregation and migration, seemingly skipping the slug phase of development altogether. These cell lines also appear to have difficulty forming fruiting bodies (Figure 11B) and whenever they do actually create stalks they tend to be much shorter on average (Figure 11C). Analysis of stalk length reveals a strong negative correlation between average stalk height and AK1 protein expression levels (Figure 11D). A two-tailed t-test performed on these data show that they are significant.

These data seem to suggest that overexpression of Alpha Kinase 1 is yielding a phenotype consistent with overexpression of other MHCKs, however the observed phenotype is not as robust as would be expected.

### **Visualization of AK1-GFP Localization *in vivo***

Additional insight into protein function can be obtained through visualization of protein localization. Being able to visualize what cellular structures the protein interacts with *in vivo* can give us greater insight into the systems or pathways that it affects within the cell. To this end, *Dictyostelium* cells were transformed with pTx-AK1-GFP allowing us to express AK1 as a GFP-tagged fusion protein. Fluorescence microscopy of these cell lines revealed a localization pattern wherein the AK1 protein appears to travel around the cell in a manner consistent with intracellular vesicular trafficking (Figure 12A). This pattern of movement seems to be unique among the other members of the MHCK family. Stimulation with cAMP does not seem to cause any significant association of GFP-tagged AK1 with the cortex of the cell (Figure 12B), nor does it appear change the localization pattern of AK1 in any appreciable manner. However, disruption of the actin cytoskeleton does change the behavior of the AK1-GFP within the cell. When treated with Latrunculin A, an inhibitor of actin polymerization, the intracellular movement of AK1-GFP appears to stop, as compared with the DMSO control treatment where the localization pattern appears to be unaffected (Figure 12C). This arresting of movement seems to support the idea that the AK1 protein is associated with intracellular vesicle trafficking.

## **CHAPTER V**

### **DISCUSSION AND CONCLUSIONS**

The broad goal of these studies was to investigate what role Alpha Kinase 1 plays in *Dictyostelium* and, more specifically, to provide evidence for its ability to act as an MHCK. Collectively, results from my thesis work suggest that Alpha Kinase 1 may play a role in regulating the turnover of myosin II filaments within *Dictyostelium*.

Immunoblot analysis of the purified AK1 protein revealed a band at approximately 230 kDa (Figure 8A). This is well above the expected protein size of 154 kDa, and could be the result of post-translational modifications, such as phosphorylation, glycosylation, etc. These might not alter the size of the protein by a significant degree, but could affect the mass-to-charge ratio enough to cause it to migrate more slowly through an SDS gel. Considering the other MHCKs have a tendency to be activated via autophosphorylation, and phosphorylation is one of the more common post-translational modifications, it seemed appropriate to investigate this as a potential explanation for why the purified AK1 protein did not run at the expected size. However, incubation with phosphatase treatment did not result in a change of apparent size of the AK1 protein. This is not necessarily indicative of the ability of AK1 to autophosphorylate, but rather seems to suggest that phosphorylation, if it occurs, does not occur to sufficient levels to alter AK1 migration. Thus, the discrepancy between expected and observed size might be due to some other modification. Another possible explanation for the size discrepancy could

be the unusual amino acid makeup of Alpha Kinase 1. Approximately 22.8% of the 1,352 amino acids are glutamine while another 13.5% are asparagine. That means that over one third of the protein is comprised of just two amino acids with polar side chains.

Furthermore, many of the glutamine residues found in Alpha Kinase 1 are strung together in very lengthy polyQ repeats. These regions could very easily affect the binding efficiency of SDS and cause problems with gel migration. Proteins that contain large amounts of acidic amino acid residues have been shown before to migrate differently than their expected size (Graceffa, et al., 1992).

A hallmark of cells overexpressing MHCKs is the presentation of a myosin II null phenotype, as an abundance of MHCK activity will force most of the intracellular myosin II into a monomeric form. A myosin II null phenotype is characterized by an inability of cells to migrate properly, undergo cytokinesis in suspension culture, or complete the developmental cycle. Thus, it follows that if the AK1 protein is acting as an MHCK, then overexpression should yield a myosin II null phenotype in *Dictyostelium*.

Analysis of the data gathered from the shaking culture assays reveals that there is a strong negative correlation with increasing levels of protein expression and growth rates. Viability, as measured using flow cytometry to detect the presence of an intact nucleus, also decreases significantly with increasing protein expression. Theoretically, cells that can grow in high levels of G418 would have high plasmid copy numbers, and since the plasmid is constitutively active, should display the myosin II null phenotype if AK1 is acting as a MHCK. Those expressing the highest amounts of protein, the G50 cell lines, display markedly decreased viabilities, with some dipping well below 50% by as

soon as day 4 in shaking culture. These characteristics are all consistent with a myosin II null phenotype, and seem to support the hypothesis that Alpha Kinase 1 is acting as an MHCK within *Dictyostelium*.

Significant levels of multi-nuclearity are observed in cell lines overexpressing AK1, as revealed by DAPI staining. By contrast, AX2 wild type cell lines and the FLAG vehicle control do not display any significant degree of multi-nucleation (it is not unusual for a small percentage of wild type cells to contain two nuclei). These data do not appear to support the idea that multi-nuclearity among cell lines increases with increasing AK1 expression, as only moderate increases in cells with large degrees of multi-nuclearity (4 nuclei or more) are observed between the G10 and G35 cell lines. The images obtained from the G50 cell lines contain less cells than the others because the cells overexpressing AK1 in such a high concentration refused to grow in shaking culture. These results demonstrate that overexpression of AK1 leads to defects in karyokinesis, which is a characteristic of the myosin II null phenotype and MHCK overexpression.

Developmental delays, another phenotype associated with MHCK overexpression, were also observed in G10, G35, and G50 cell lines. Normal *Dictyostelium* development occurs within 24-48 hours after the introduction of stressful environmental conditions, such as starvation. Development follows several distinct phases: aggregation, migration, and culmination. In healthy, wild type cells, aggregation generally begins 4-8 hours into development, with migration beginning around 12-16 hours and culmination lasting up to 24 hours or more. From my trials, the AX2 wild type and the FLAG control cells seem to follow this trend very closely. By comparison, the



G10, G35, and G50 experimental groups display large delays in development. These cell lines begin aggregation nearly 12 hours into development, a full 8 hours later than the wild type control. These cell lines also seem to sometimes skip the slug phase that is present in the AX2 wild type control and aggregate directly into mounds, where they form stalks sometimes as late as 12 hours after the control groups. There also seems to be a negative correlation between protein expression and stalk length, as revealed by my data. Cells expressing the highest levels of the AK1 protein form the shortest stalks. This is most likely due to the developmental delays associated with overexpression of the AK1 protein, however it does not rule out the possibility that the AK1 protein might somehow be explicitly implicated in proper stalk formation. It is interesting to note that while the G10, G35, and G50 cell lines all appeared to reach the mound stage at the same time (roughly 16 hours into the experiment), they displayed markedly differing stalk heights after 48 hours. These results differ slightly from typical results obtained from the overexpression of MHCKs, as overexpression of MHCKs tends to result in arrested development at the mound stage. Cell lines overexpressing AK1 do sometimes halt development at the mound stage, but this does not appear to always be the case. Still, the fact that overexpression of AK1 is causing significant delays early in development seems to suggest that it has an impact on cell motility, a defect associated with the myosin II null phenotype and MHCK overexpression. Taken together, these data seem to suggest that AK1 is acting in some way to influence turnover of myosin II filaments within *Dictyostelium*.

The localization studies revealed a very interesting phenotype, something that appears to be unique amongst the alpha kinase family in *Dictyostelium*. The other MHCKs in *Dictyostelium* have very distinct localization patterns that correspond with their intracellular functions. For example, MHCK A localizes to the leading edge of the cell where it is involved in regulating actin filament turnover to allow the cell to extend in that direction, whereas MHCK C localizes to the rear of the cell where it helps to regulate the formation of actin filaments to contract the trailing end to help propel the cell forward. Alpha Kinase 1 appears to do neither of these things, and instead forms compact pockets of protein that appear to travel around the cell in a manner consistent with vesicle trafficking. This idea is further supported by the fact that upon treatment with Latrunculin A, a compound which breaks down the actin cytoskeleton, the AK1-GFP fusion protein appears to stop moving. Vesicles travel along the actin cytoskeleton within cells to arrive at their destination (usually the cell membrane). However, due to the unique primary structure of AK1, these localization patterns could also be due to the formation of large protein aggregates. If this were the case, then upon treatment with Latrunculin A, we would also expect to see these aggregates stop moving. In order to definitively say what is causing this unique localization pattern, live staining of *Dictyostelium* using an anti-AK1 antibody will need to be performed.

Stimulation of the cells with cAMP causes no discernable effects either, in contrast with the localization profiles of other known MHCKs. Depending on their function, MHCKs will become enriched at different parts of the cell upon stimulation

with cAMP. Because this behavior is not seen in Alpha Kinase 1, it could indicate that it is not responsible for direct phosphorylation of myosin II heavy chain.

This interesting behavior makes sense when considering the unique makeup of the Alpha Kinase 1 protein, as it contains only two known domains: an alpha kinase catalytic domain and an ARF-GAP domain. ARFs (ADP ribosylation factors) are a highly conserved class of GTP binding proteins, part of the much larger superfamily of Ras proteins, and are found amongst almost all eukaryotes. ARFs function as regulators of both actin remodeling and vesicular trafficking and are found to be associated most often with the extracellular membrane or organelles such as the golgi apparatus (Randazzo and Hirsch, 2004). It would follow then that Alpha Kinase 1, since it contains one of these ARF-GAP domains, could be associated so strongly with vesicles. However, a more in-depth investigation of these localization patterns will be needed to determine if these results are truly indicative of vesicle association and not just an artifact of protein aggregates. Interestingly enough, while humans have 6 separate classes of ARF proteins, there is only one known *Dictyostelium* ARF. To date, there have been relatively few studies concerning the function of ARFs within *Dictyostelium* so it would be hard to speculate exactly what role the ARF-GAP domain found in the Alpha Kinase 1 protein would have in its overall function within the cell outside of very generalizing hypotheses. However, based off what we know about human ARFs, it seems very likely that Alpha Kinase 1 must be implicated in some sort of cytoskeletal remodeling either by directly influencing myosin II filament turnover through its alpha kinase catalytic domain or indirectly by inducing hydrolysis of ARF-bound GTP.

Another possible insight into the function of Alpha Kinase 1 can be found in the protein structure itself. The Alpha Kinase 1 protein contains numerous, lengthy polyQ regions. Long stretches of repeating glutamine residues have been implicated in numerous types of protein-protein interactions (Schaefer et al., 2012). This could imply a more indirect, regulatory role in myosin II filament turnover. If this were the case, then this could explain many of the inconsistencies observed in the myosin II null phenotype displayed as a result AK1 overexpression. If AK1 were acting through the other MHCKs, either directly or through some secondary signaling pathway, then these interactions could very well be rate-limiting steps.

A more in-depth exploration of the enzymatic capabilities of AK1 is needed to truly determine its primary function within the cell. My data has shown that the AK1 protein can be successfully purified. Future studies could directly investigate the enzymatic activity of its alpha kinase catalytic domain. The localization data I have presented here could be expanded upon as well by looking for co-localization of AK1 and other MHCKs or ARFs within the cell. Over-expression of AK1 in cell lines expressing a MyoII-GFP fusion protein would allow for great insight into how AK1 visibly affects myosin II organization *in vivo*. Generation of AK1-null cell lines would also be a great next step for future studies, as protein knockout studies can give great information regarding protein function *in vivo*. Overexpressing AK1 in MHCK A<sup>-</sup>B<sup>-</sup>C<sup>-</sup> mutants (cell lines lacking the other MHCKs) could also give us insight into whether or not AK1 is playing a direct or indirect role in influencing myosin II filament turnover. Cell lines lacking the other MHCKs display a phenotype associated with over-assembly of myosin

II. If overexpression of AK1 in these cell lines rescues the phenotype, then this could indicate that AK1 is acting in some way to directly influence myosin II filament turnover.

In conclusion, more data will be needed to determine if Alpha Kinase 1 is a true MHCK, but I am confident in saying that my data supports the hypothesis that Alpha Kinase 1 is acting in some way to influence cytoskeletal remodeling within *Dictyostelium discoideum*.

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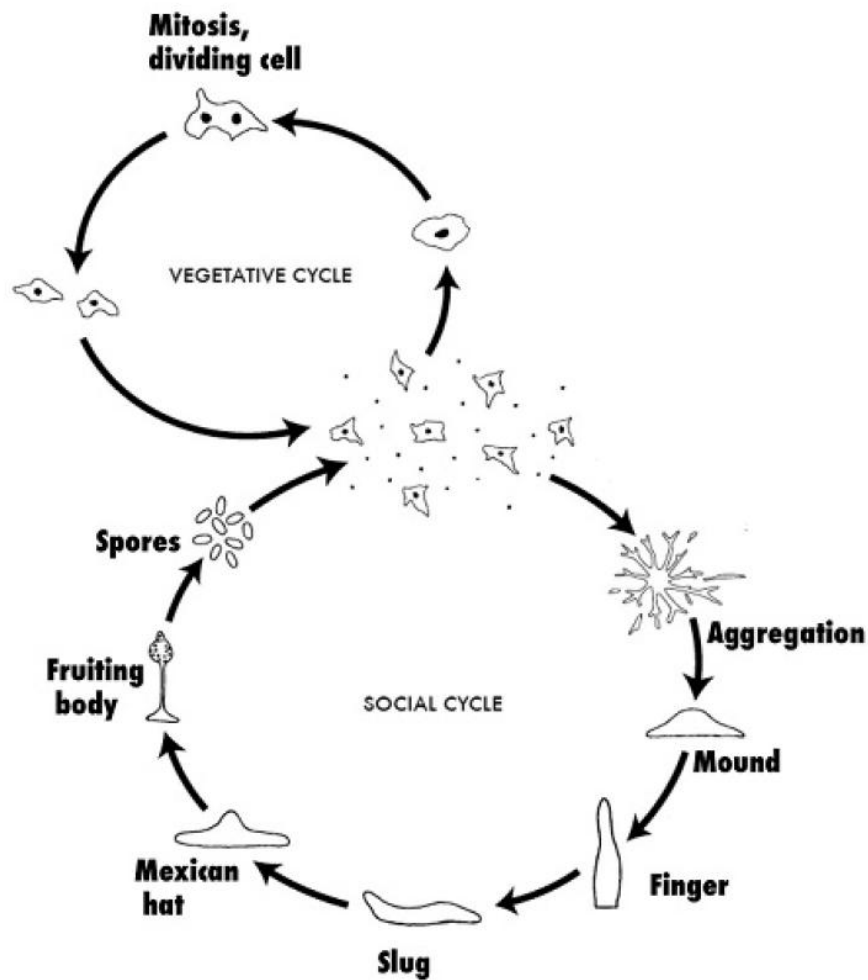
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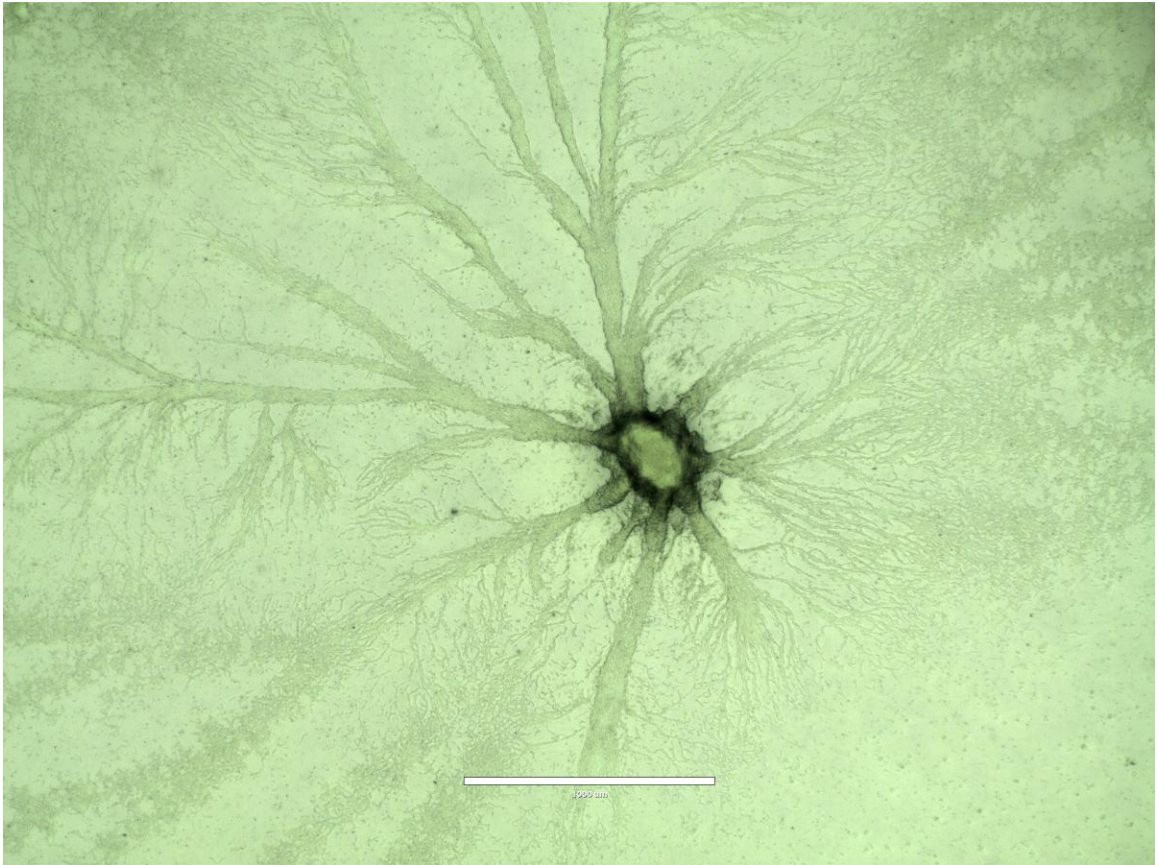
## APPENDIX A

### FIGURES

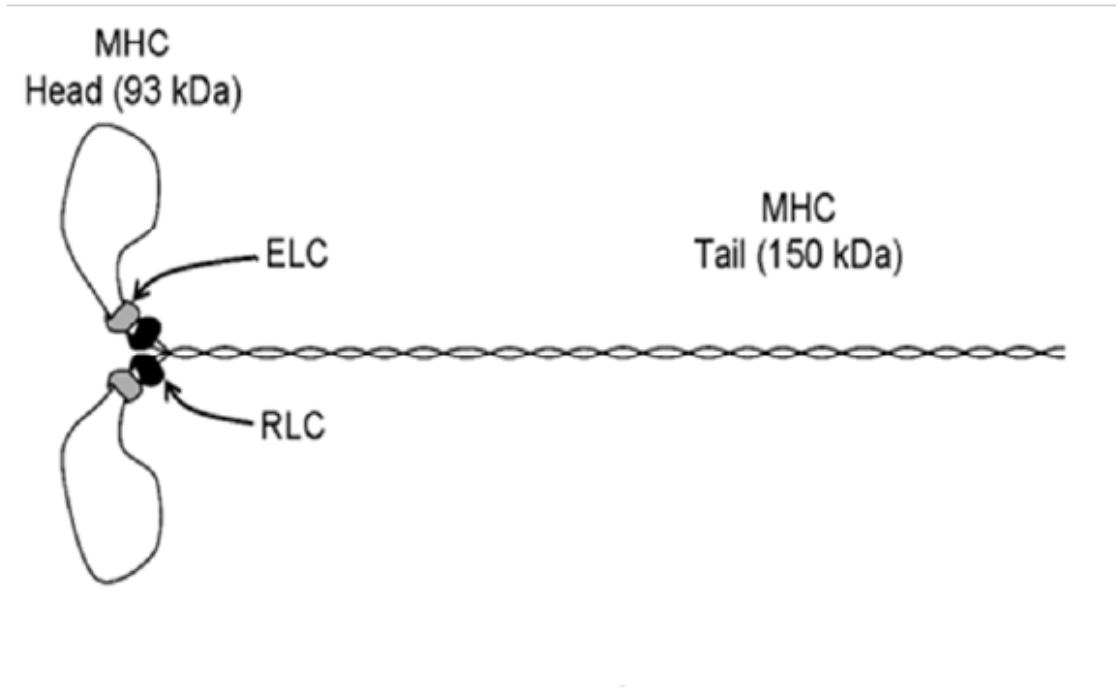


**Figure 1. Life Cycle of *Dictyostelium discoideum***

*Dictyostelium* alternates between a free-living, vegetative state and a multicellular, developmental state depending upon environmental conditions.

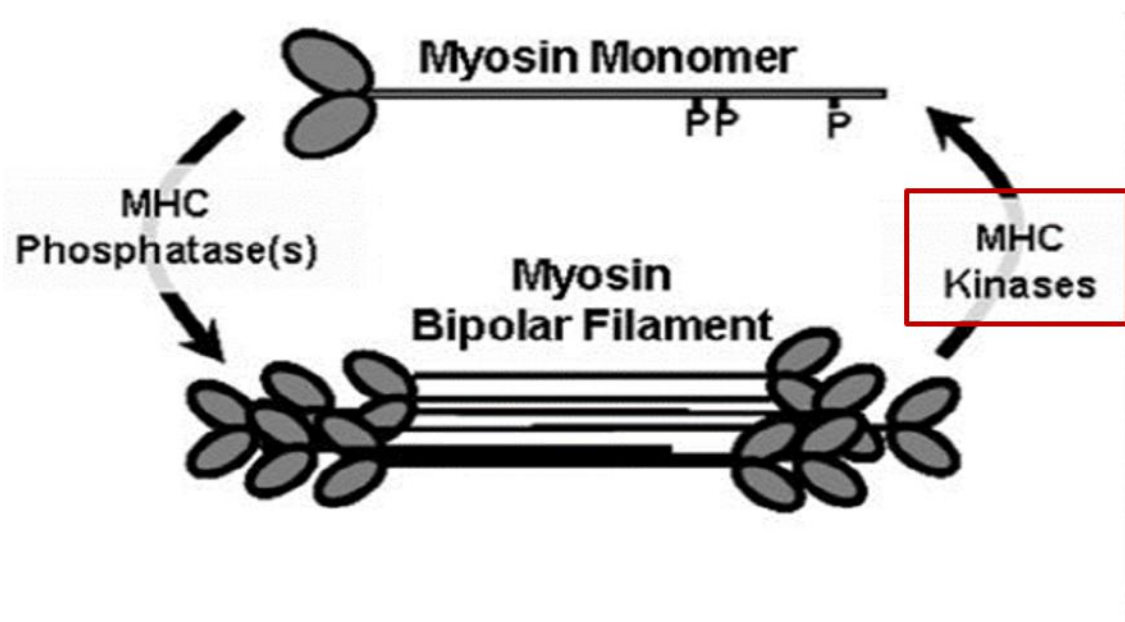


**Figure 2. Aggregation Phase in the Developmental Cycle of *Dictyostelium discoideum***  
Under stressful environmental conditions, *Dictyostelium* undergo a transition to a multicellular state. This transition begins with aggregation, where cells migrate up a cAMP concentration gradient that it established by a “founder cell.” This process ultimately leads to the formation of a mound and eventually a fruiting body containing spores.



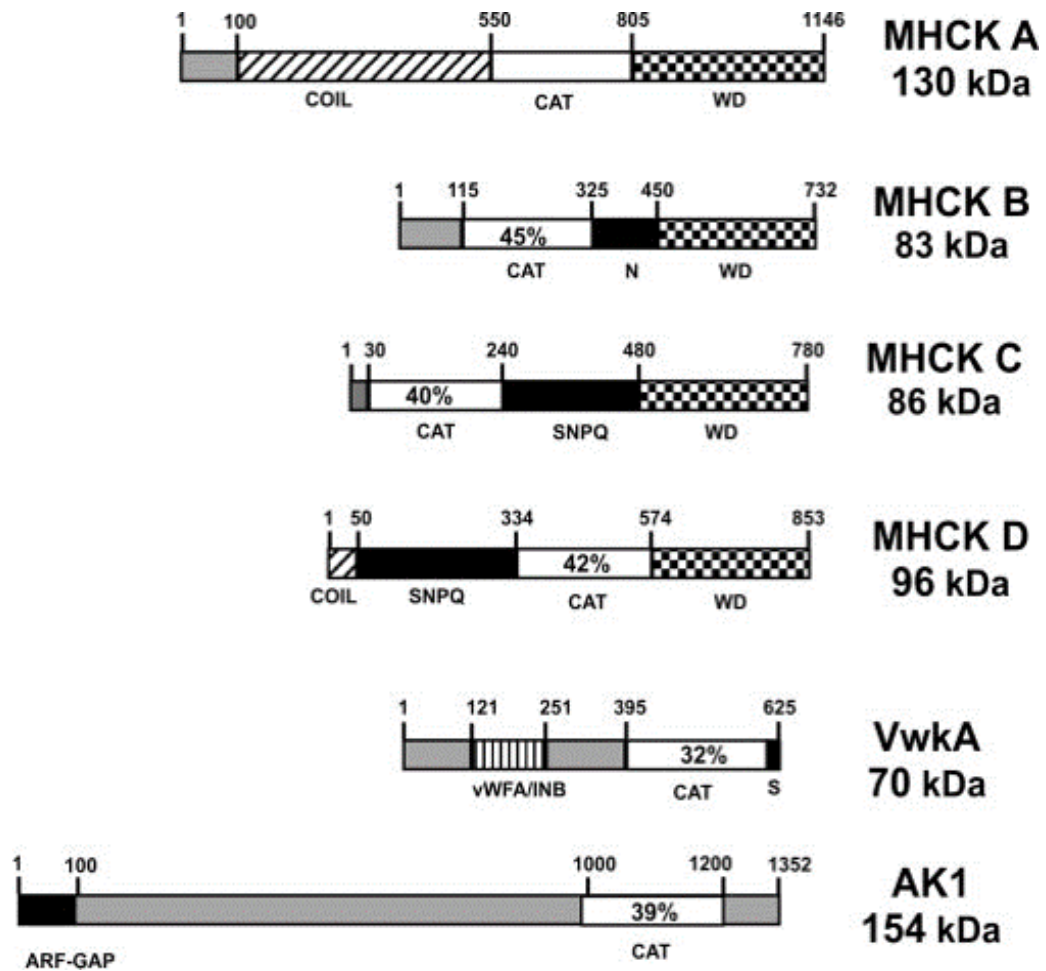
**Figure 3. Diagram of Myosin II**

Myosin II is hexameric protein comprised of two essential light chains, two regulatory light chains, and two heavy chains. In *Dictyostelium*, myosin II associates with the actin cytoskeleton and is directly responsible for the contraction of opposing actin filaments using the energy derived from ATP hydrolysis. Transition between a monomeric state and a filamentous state is driven by the phosphorylation or dephosphorylation of three specific threonine residues on the heavy chain tail.



**Figure 4. MHCKs Regulate Myosin II Assembly in *Dictyostelium***

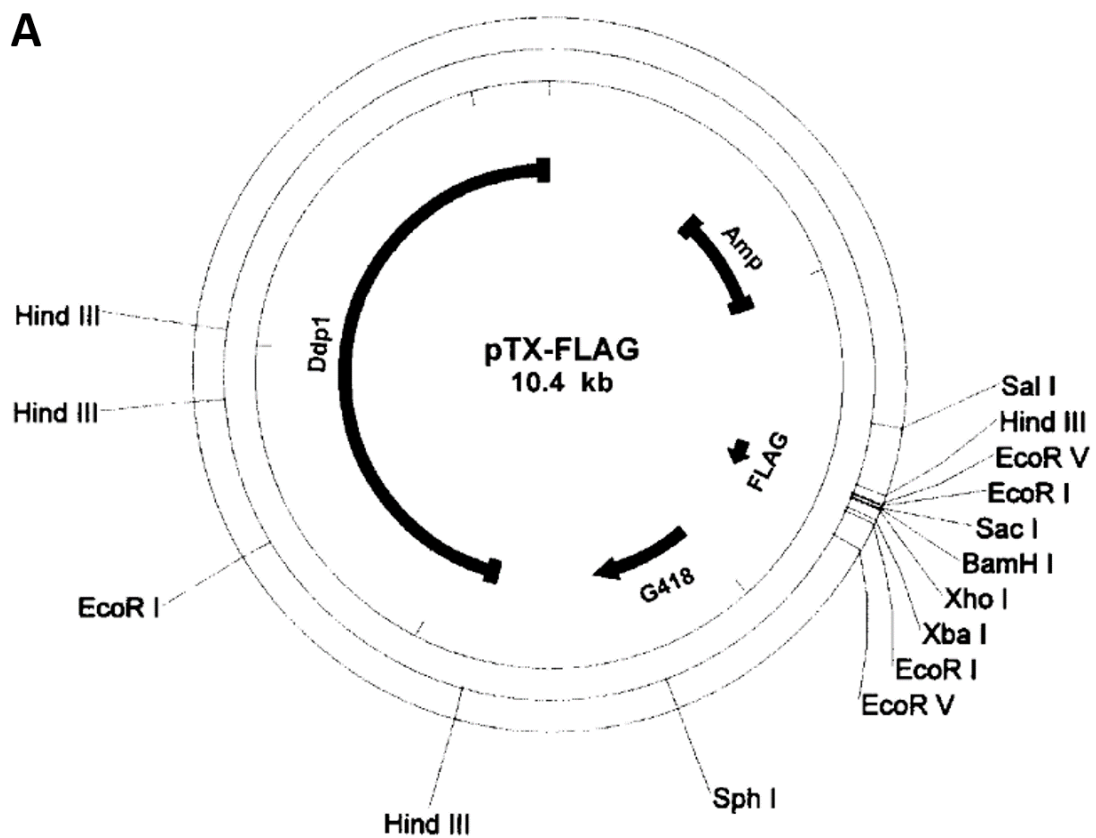
Myosin heavy chain kinases are directly responsible for driving the transition of myosin II from a filamentous state to a monomeric state. Phosphorylation of the myosin heavy chain causes disassembly of myosin bipolar filaments into myosin II monomers. In this state, myosin II is unable to contract actin and, by extension, contraction of the cell cortex cannot occur.



**Figure 5. Diagram of the Alpha Kinase Family in *Dictyostelium***

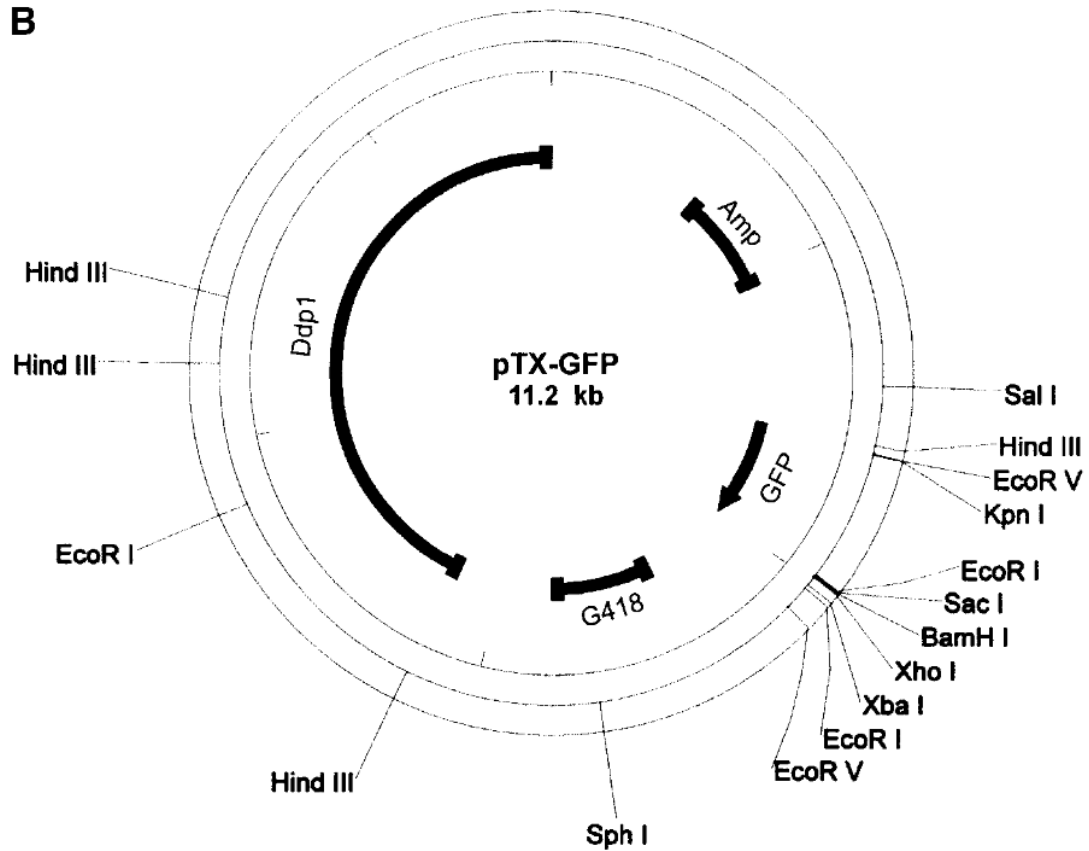
Proteins within this family all share an alpha kinase catalytic domain as a part of their overall structure. Only MHCKs A through D have been demonstrated to directly phosphorylate myosin II.





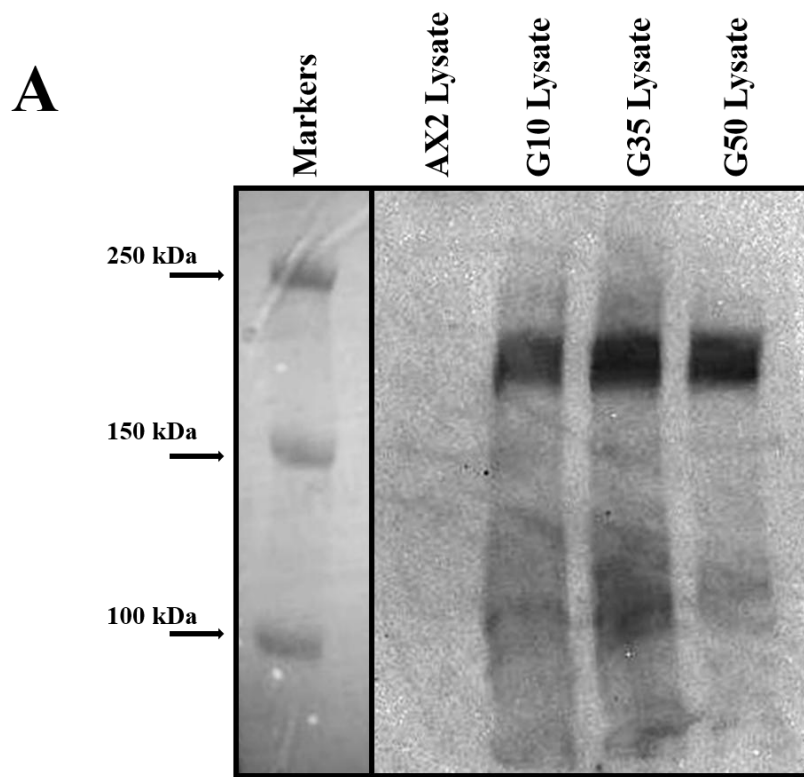
**Figure 7A. Diagram of pTx-FLAG Plasmid**

Schematic representation of the plasmid that will allow for AK1 over-expression. It contains an actin15 promoter and a G418 resistance region. Image courtesy of Levi, S., Polyakov, M., & Egelhoff, T. T. (2000). Green fluorescent protein and epitope tag fusion vectors for *Dictyostelium discoideum*. *Plasmid*, 44(3), 231-238.

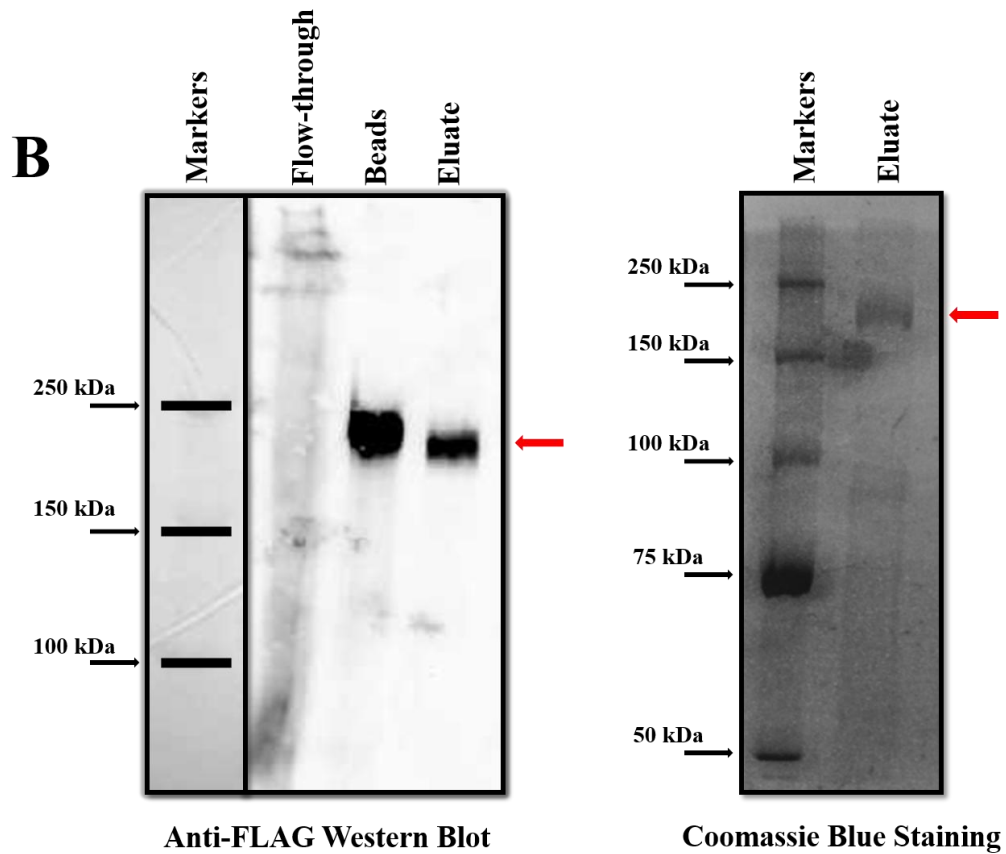
**B****Figure 7B. Diagram of pTx-GFP Plasmid**

Schematic representation of the plasmid that will allow for visualization of AK1 localization *in vivo*. It contains an actin15 promoter and a G418 resistance region. Image courtesy of Levi, S., Polyakov, M., & Egelhoff, T. T. (2000). Green fluorescent protein and epitope tag fusion vectors for Dictyostelium discoideum. *Plasmid*, 44(3), 231-238.



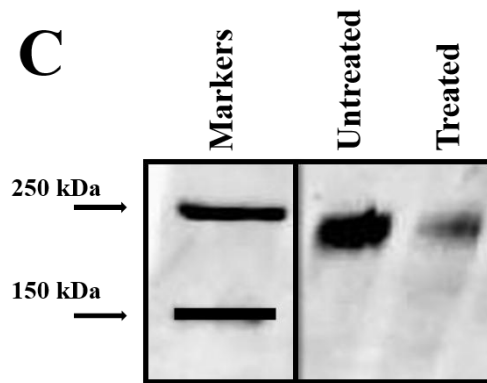


**Figure 8A. Western Blot Analysis of Cell Lines Overexpressing Alpha Kinase 1**  
Lysates prepared from AX2 wildtype, G10, G35, and G50 cell lines. Blot was probed with anti-FLAG antibody.



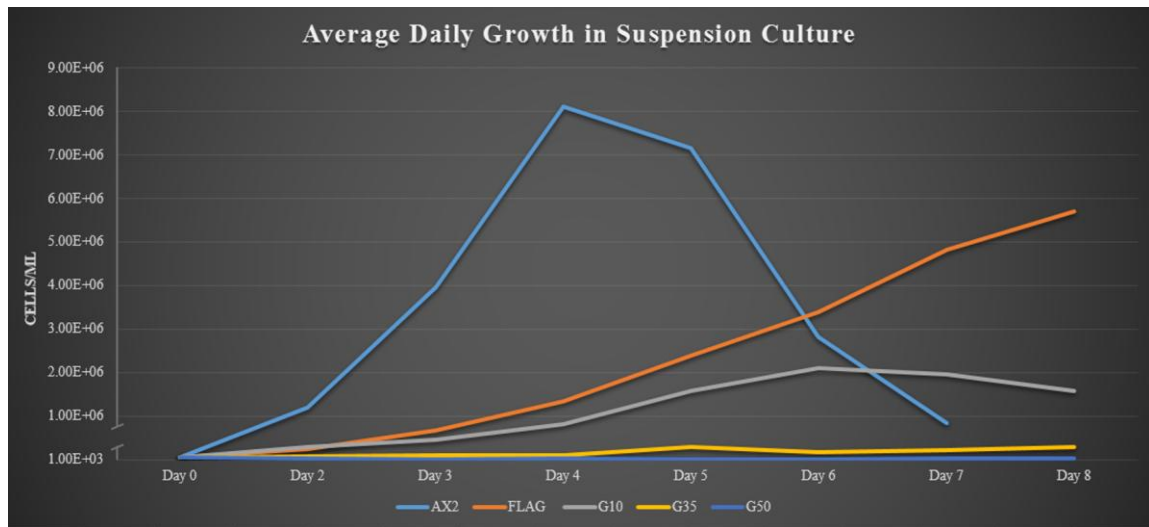
**Figure 8B. Affinity Purification of Alpha Kinase 1**

The Alpha Kinase 1 fusion protein was successfully purified from G50 cell lines using affinity purification. Samples from the flow-through indicate that nearly 100% of the protein in the lysates was successfully bound to the beads, although much of that remains after the elution step. There is no breakdown detected in the sample from the eluate. Coomassie blue staining reveals only one band at approximately the same size.



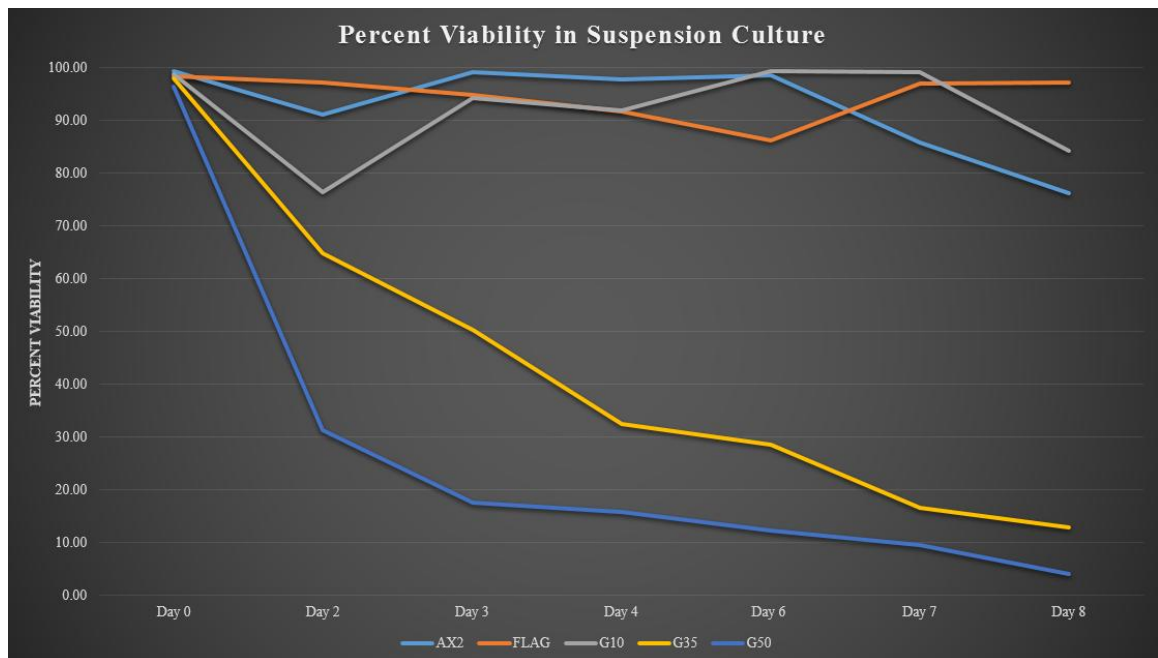
**Figure 8C. Treatment of Alpha Kinase 1 with Antarctic Phosphatase**

Western blot probed with anti-FLAG antibody. After incubation with Antarctic Phosphatase, the protein did not appear to change size. This indicates that phosphorylation was not the reason for the apparent discrepancy between expected and observed size.



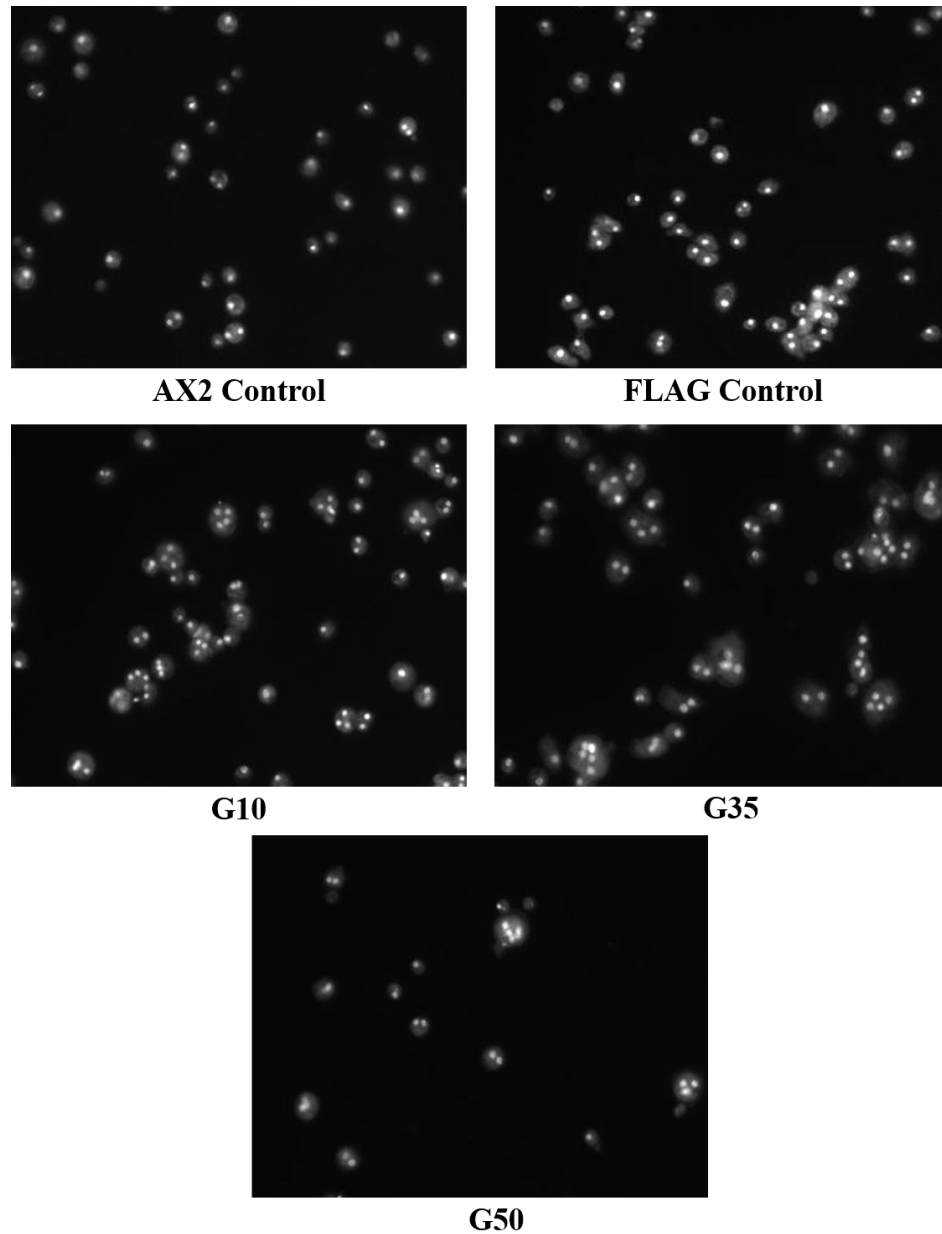
**Figure 9A. Average Daily Growth in Suspension Culture**

Cells overexpressing Alpha Kinase 1 grew poorly in shaking culture as compared with AX2 wildtype controls and FLAG vehicle controls.



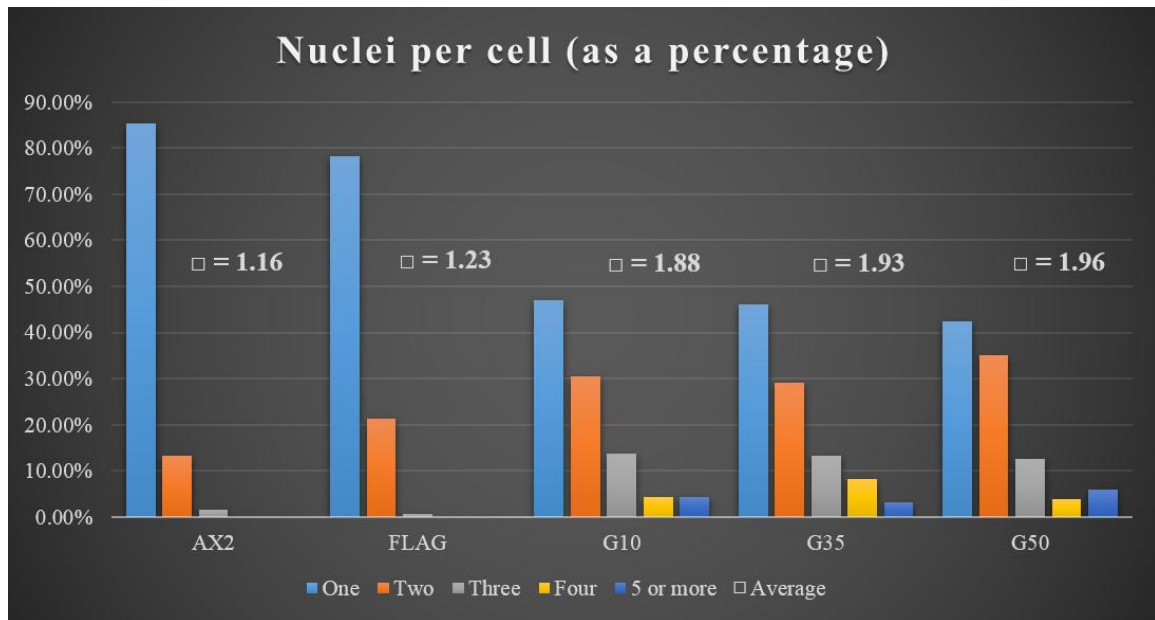
**Figure 9B. Viability in Suspension Culture**

This graph illustrates the overall viability of each cell line in suspension culture. Viability was determined by the intactness of the nucleus. There is a very large decrease in viability for the G35 and G50 cell lines after only a few days in suspension culture.



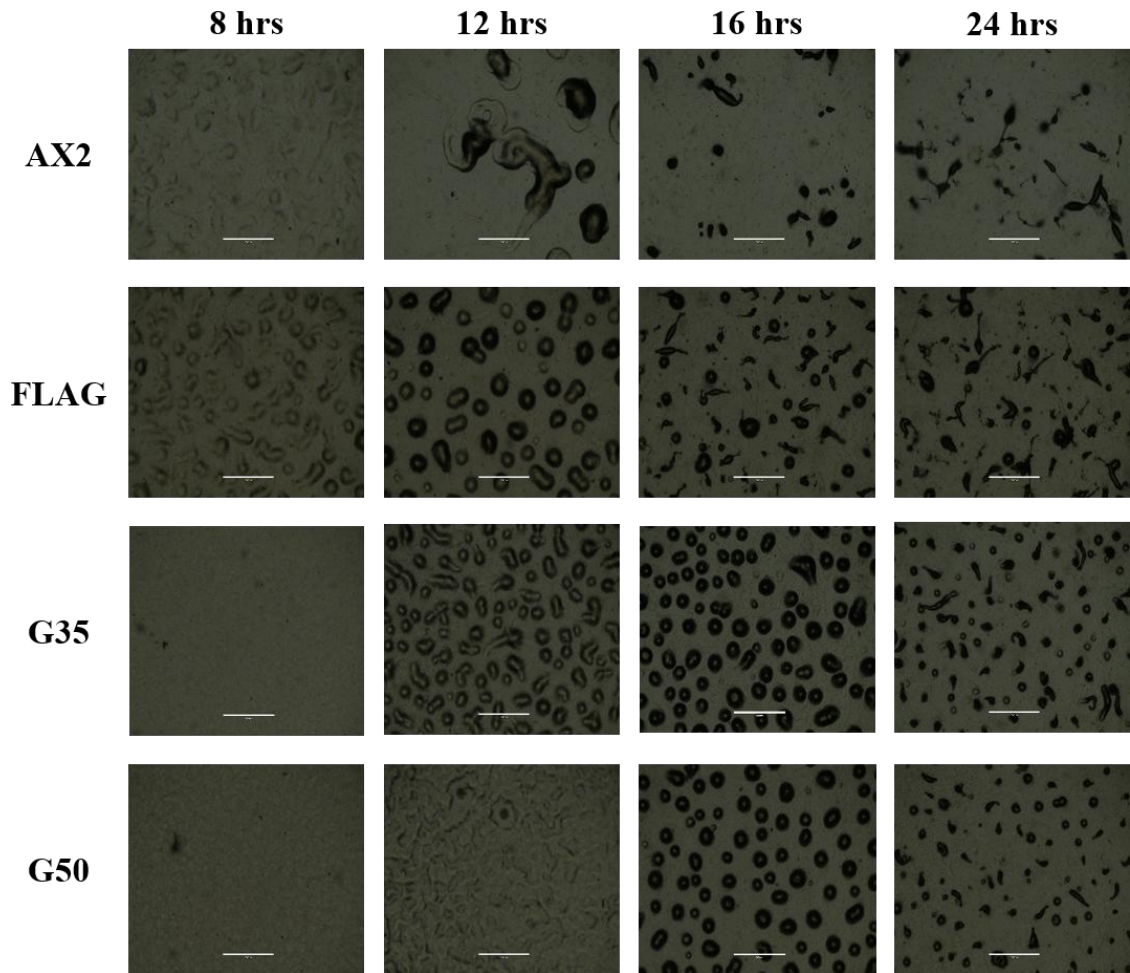
**Figure 10A. DAPI Staining of Cell Lines Overexpressing Alpha Kinase 1**

DAPI staining revealed significant multi-nucleation in cell lines overexpressing AK1. The images obtained from the G50 cell lines show them to contain significantly less cells than the others. Poor growth in suspension culture is responsible for this.



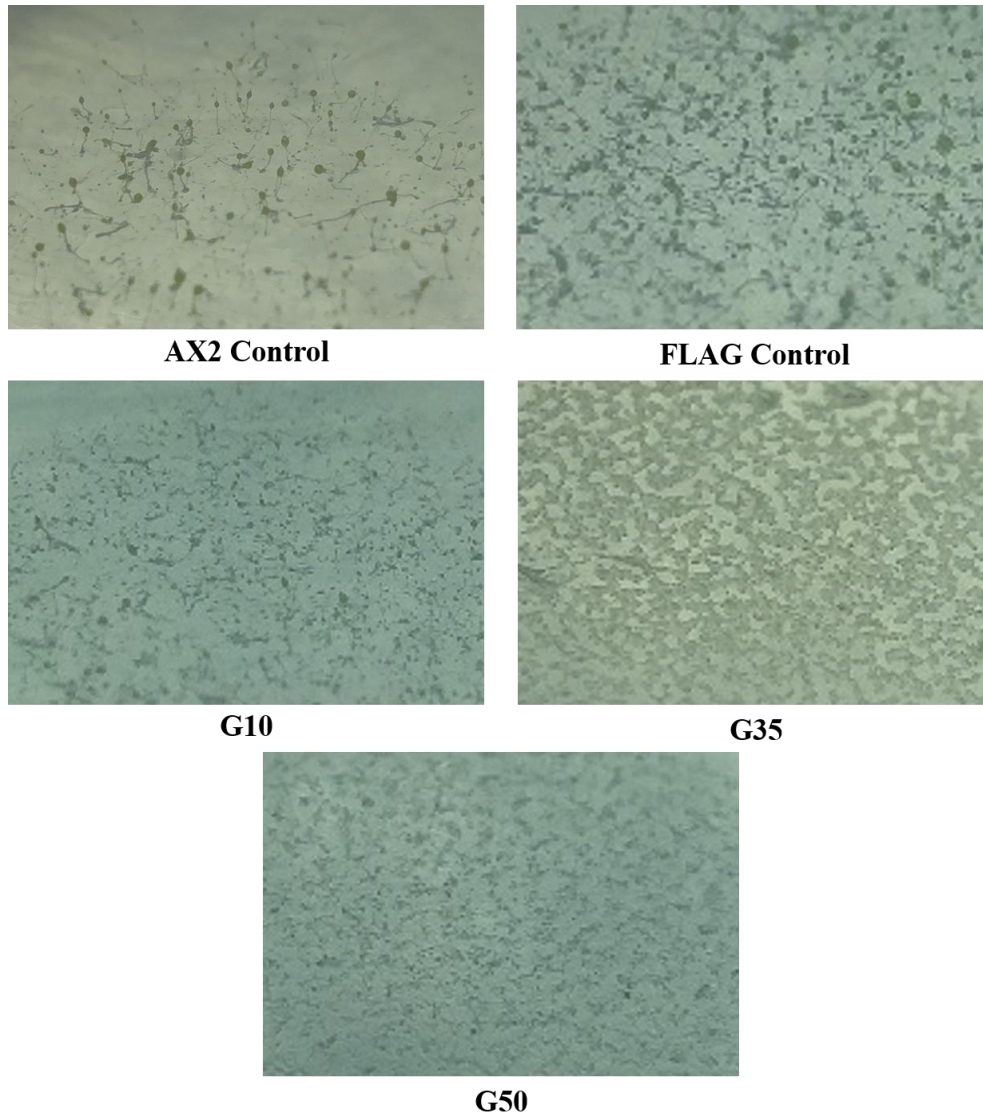
**Figure 10B. Alpha Kinase 1 Overexpression Promotes Multi-nucleation**

Quantification of the images obtained from DAPI staining indicate that AK1 overexpression is strongly correlated with multi-nucleation in shaking culture. The average nuclei per cell shows a nearly 60% increase in the G50 cell lines over the AX2 control.



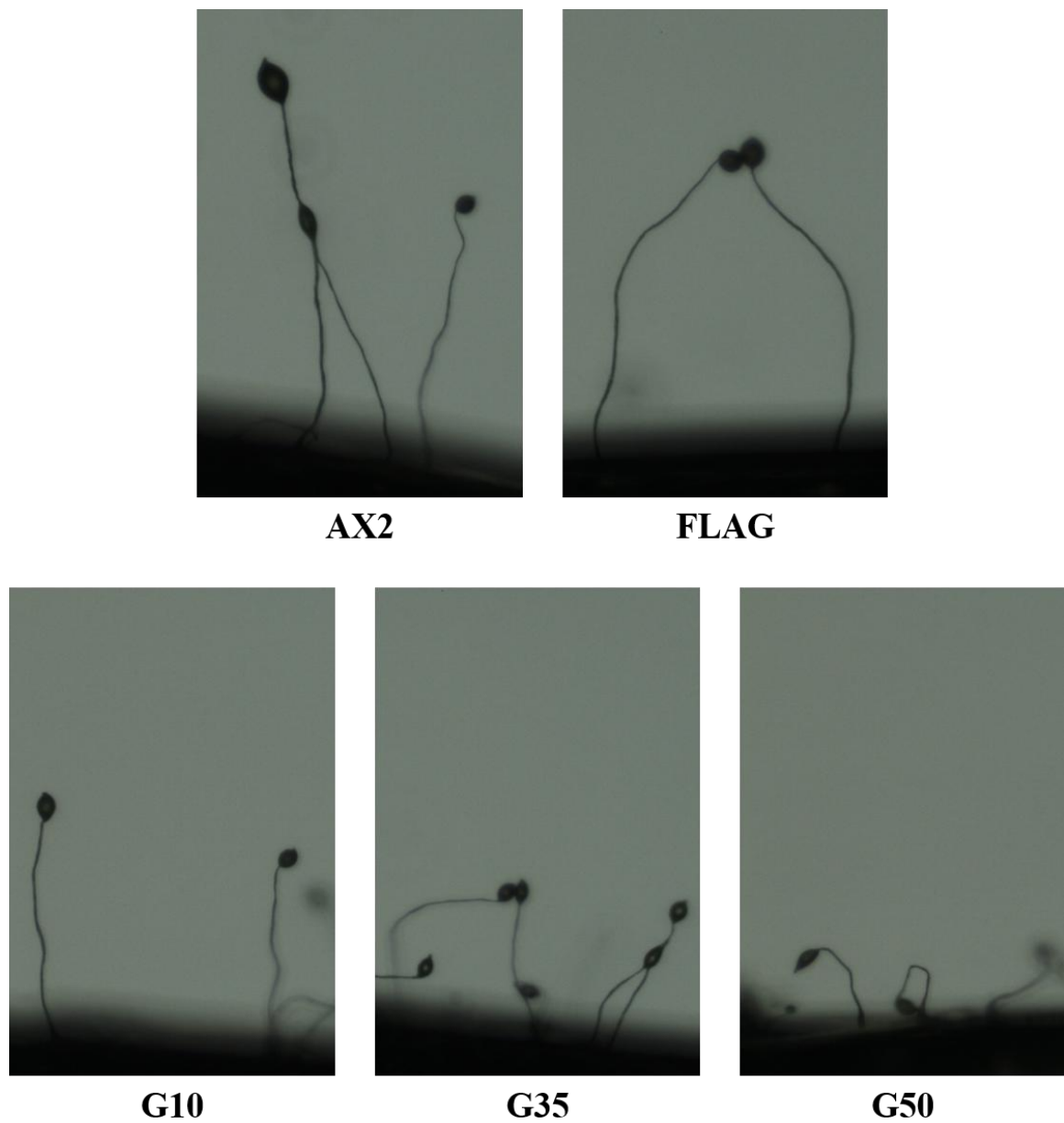
**Figure 11A. Developmental Delays Associated with Alpha Kinase 1 Overexpression**  
 Cell lines overexpressing AK1 show delays in the early stages of development, particularly during aggregation. The AX2 wild type control and the FLAG control both reach the mound phase after roughly 8 hours into development and progress to the migratory or slug phase by 12 hours. By comparison, the G35 and G50 cell lines take up to 12 hours to reach the mound phase and don't reach the migratory phase until almost 24 hours after the start of development.





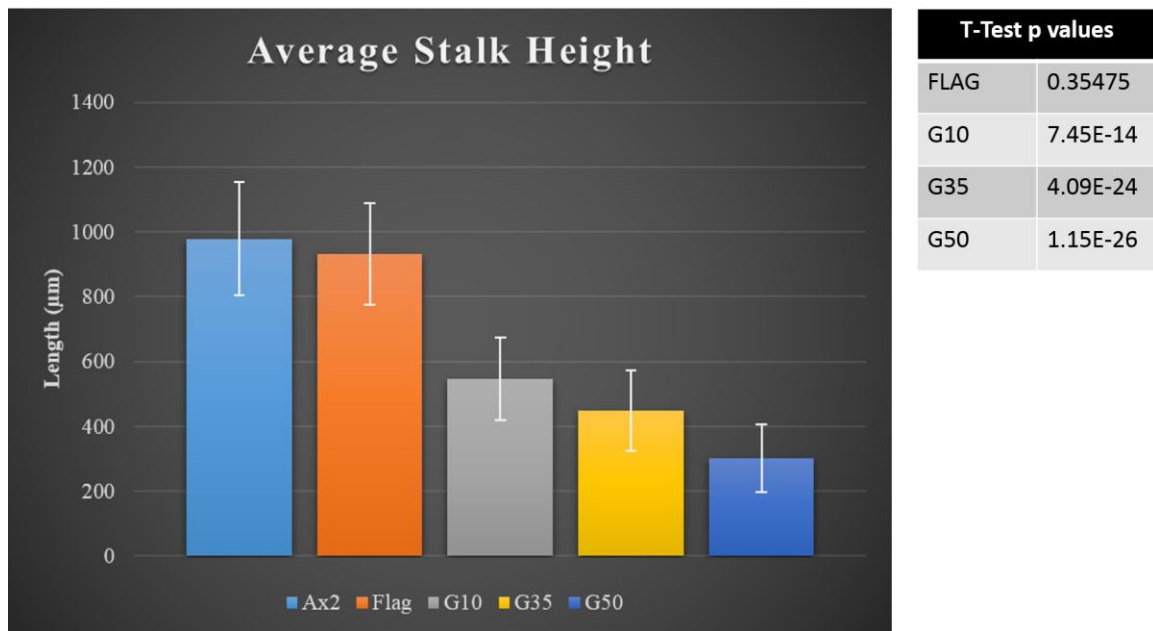
**Figure 11B. Alpha Kinase 1 Overexpression Results in Incomplete Formation of Fruiting Bodies**

Cell lines overexpressing AK1 tend to halt development at the mound stage, a hallmark characteristic of MHCK overexpression. Images were taken at 48 hours after the start of development.



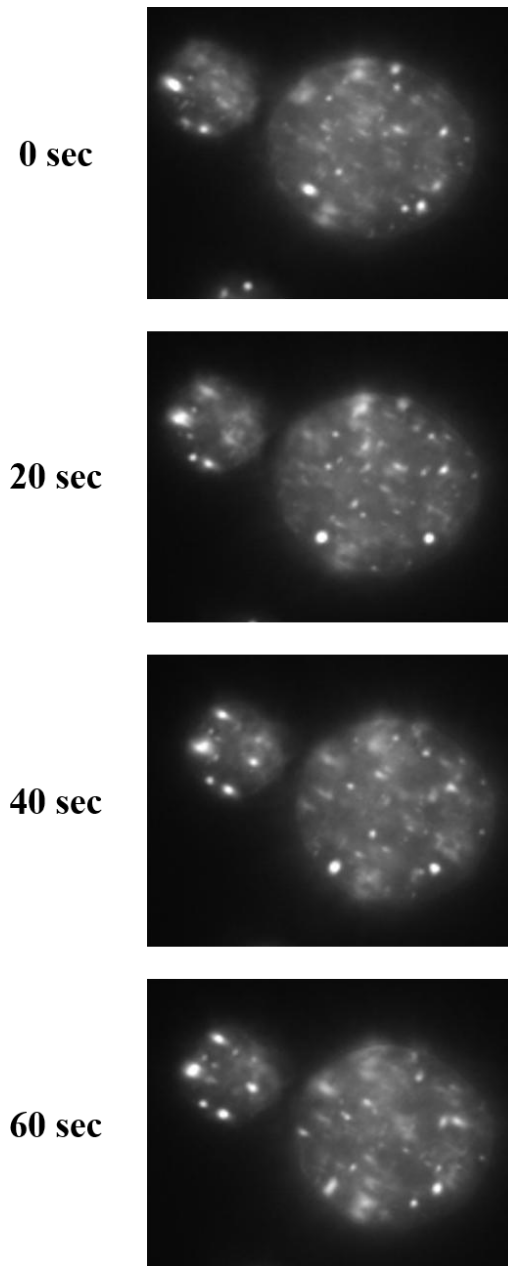
**Figure 11C. Comparison of Stalk Heights in Developing Cells Overexpressing Alpha Kinase 1**

In developing cell lines that do manage to produce fruiting bodies, stalk height is significantly shortened. If plated in new media, the spores remain viable. Images taken 48 hours after the start of development.



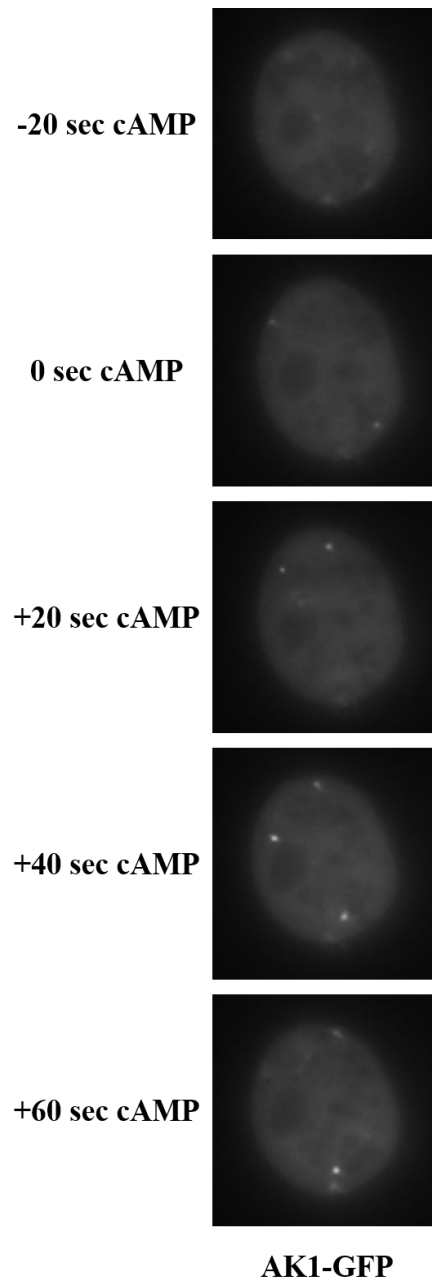
**Figure 11D. Average Stalk Length is shorter in Cell Lines Overexpressing Alpha Kinase 1**

When fruiting bodies do form, the stalks tend to be shorter on average for cell lines overexpressing Alpha Kinase 1. G50 cell lines in particular display a markedly reduced stalk height that is well below the average wildtype stalk height. Heights did not vary considerably between the two controls, AX2 and FLAG cell lines. A two-tailed T-test conducted between each trial and the AX2 wildtype control revealed that the differences in stalk height between all three experimental groups was highly significant.



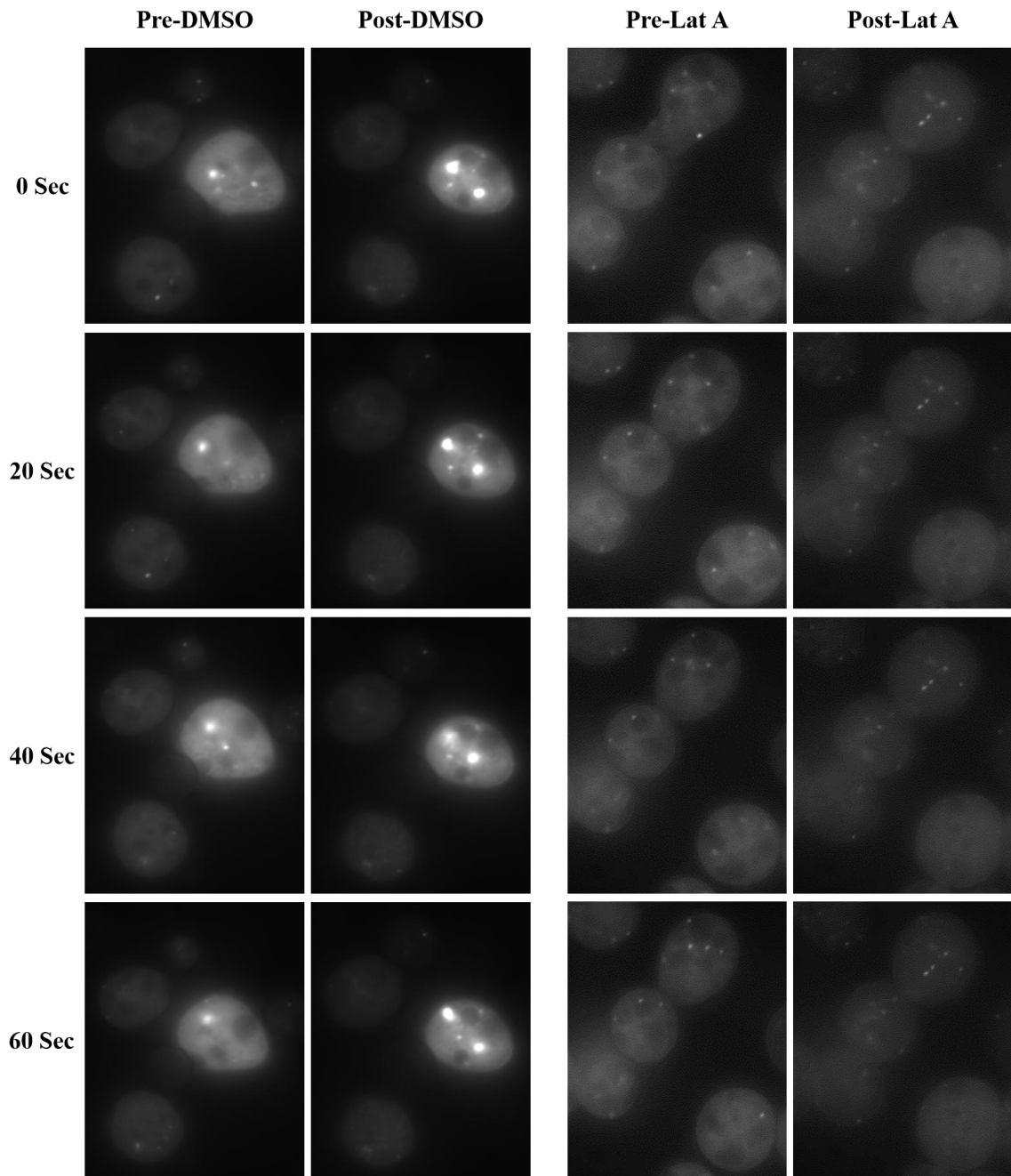
**Figure 12A. Localization Patterns of Alpha Kinase 1**

GFP-tagged Alpha Kinase 1 demonstrates a unique localization pattern among the members of the alpha kinase family. It presents as small pockets of protein that appear to travel around the cell in a manner consistent with vesicle trafficking.



**Figure 12B. Stimulation with cAMP Does Not Affect Intracellular Localization of Alpha Kinase 1**

In cells expressing GFP-tagged AK1, stimulation with cAMP does not seem to cause any discernable change in localization. This is in contrast with other MHCKs that appear to enrich at different parts of the cell during cAMP stimulation.



**Figure 12C. Treatment with Latrunculin A Arrests Intracellular Movement of Alpha Kinase 1**

When cells expressing the AK1-GFP fusion protein are treated with Latrunculin A, an inhibitor of actin polymerization, the intracellular movement patterns of AK1 appear to halt. Cells treated with DMSO were used as a control.